COVER LEGEND

Duckbill Platypus, painted by San Francisco Bay artist, Pat Sherwood. As the most ancestral mammalian relatives of humans, the egg-laying monotremes, the platypus and echidna, offer insight into events that occurred at the dawn of mammalian evolution. Killian et al. demonstrate in this issue that IGF2 is not imprinted in monotremes, providing the first compelling evidence that the phylogenetic breadth of this epigenetic gene regulatory phenomenon is restricted to marsupials and placental mammals.
Monotreme IGF2 Expression and Ancestral Origin of Genomic Imprinting

J. KEITH KILLIAN,1 CATHERINE M. NOLAN,2 NIA LL STEWART,3 BARRY L. MUNDAY,3 NIELS A. ANDERSEN,4 STEWART NICOL,4 AND RANDY L. JIRTLE1*

1Departments of Radiation Oncology and Pathology, Duke University Medical Center, Durham, North Carolina 27710
2Department of Zoology, University College Dublin, Ireland
3School of Biomedical Science, University of Tasmania, Launceston, Tasmania 7250, Australia
4Discipline of Anatomy and Physiology, University of Tasmania, Hobart, Tasmania 7001, Australia

ABSTRACT IGF2 (insulin-like growth factor 2) and M6P/IGF2R (mannose 6-phosphate/insulin-like growth factor 2 receptor) are imprinted in marsupials and eutherians but not in birds. These results along with the absence of M6P/IGF2R imprinting in the egg-laying monotremes indicate that the parental imprinting of fetal growth-regulatory genes may be unique to viviparous mammals. In this investigation, we have cloned IGF2 from two monotreme mammals, the platypus and echidna, to further investigate the origin of imprinting. We report herein that like M6P/IGF2R, IGF2 is not imprinted in monotremes. Thus, although IGF2 encodes for a highly conserved growth factor in chordates, it is only imprinted in therian mammals. These findings support a concurrent origin of IGF2 and M6P/IGF2R imprinting in the late Jurassic/early Cretaceous period. The absence of imprinting in monotremes, despite apparent interparental conflicts over maternal–offspring exchange, argues that a fortuitous congruency of genetic and epigenetic events may have limited the phylogenetic breadth of genomic imprinting to therian mammals. J. Exp. Zool. (Mol. Dev. Evol.) 291:205–212, 2001. © 2001 Wiley-Liss, Inc.
In monotremes and birds (Killian et al., 2000; Nolan et al., 2001). We also discovered that like birds, platypus M6P/IGF2R does not bind IGF2 and therefore does not regulate embryonic growth in monotremes. Because receptor imprinting may be related to its ability to regulate embryonic growth, this functional divergence of M6P/IGF2R makes it noninformative for tracing the origin of imprinting beyond therian mammals. Furthermore, unlike most imprinted genes, M6P/IGF2R imprinting is divergent in therian mammals. Imprint analysis in monotremes must therefore be extended beyond M6P/IGF2R to more precisely determine the phylogenetic origin of imprinting.

IGF2 is structurally conserved throughout chordates, and gene imprinting is universal in analyzed therian mammals. Although IGF2 is imprinted in both marsupials and eutherians, we report herein that it is not imprinted in monotremes. The overall pattern of M6P/IGF2R and IGF2 imprinting in birds, monotremes, marsupials, and eutherians indicates a brusque origin of genomic imprinting in Jurassic/Cretaceous therian mammals following their divergence from prototherians. The discrepant IGF2 imprinting between monotremes and marsupials, despite significant reproductive similarities, suggests that genetic and epigenetic convergences required for locus imprinting limited its phylogenetic breadth.

MATERIALS AND METHODS

Tissue samples

Tasmanian and mainland Australian platypus (Ornithorhynchus anatinus) visceral organs (i.e., kidney, liver, and spleen) and skin biopsies were obtained from wild animals that were a victim of dog attack and were under surveillance, respectively. Echidna (Tachyglossus aculeatus) tissues (i.e., intestine, kidney, liver, and spleen) were harvested from animals that were accidentally killed in Tasmania by automobiles. All samples were transported in either dry ice or RNAlater (Ambion, Austin, TX) from the University of Tasmania to Duke University where they were maintained at −80°C until DNA and RNA extraction.

Isolation and sequencing of platypus and echidna IGF2

Total RNA was isolated from platypus and echidna tissues by homogenization in RNA-Stat 60 (Tel-Test, Friendswood, TX), and subsequent processing was performed as recommended by the manufacturer. First-strand cDNA was synthesized from 1–5 μg of DNase-treated RNA using Super-
Script II as recommended by the manufacturer (Life Technologies, Baltimore, MD). Four nondegenerate cross-species IGF2 primers were designed based upon sequence alignment of available orthologues in GenBank. A highly conserved region of IGF2 was successfully amplified from cDNA with four primer combinations consisting of the forward primer CS-IGF2F1 (5'-CGGCGGGAGCTGTGACGAC) or the forward primer CS-IGF2F2 (5'-TGGGGACCGCGGCTTACTTCACG) and the reverse primer CS-IGF2R1 (5'-GACTTGCGGGGTTGCCACAG) or the reverse primer CS-IGF2R2 (5'-GGGGTGGCACAGTACGTCTCCAG) using 1.5 U Platinum Taq DNA polymerase (Life Technologies, Baltimore, MD), 15 pmol of primers, 1.5 mM MgCl₂, and 100 μM dNTPs in a 30-μl PCR reaction volume (94°C × 15 sec, 55°C × 5 sec, and 72°C × 45 sec for 30–35 cycles). RT-PCR products were analyzed by electrophoresis on a 2.0% agarose gel, and the appropriately sized fragments were excised and gel-extracted (GenElute, Sigma Chemical Co., St. Louis, MO). Following direct sequencing of these RT-PCR products (ABI 377 sequencer; PE Biosystems, Foster City, CA), the complete monotreme IGF2 sequence was ascertained with the use of gene-specific primers in 3'-RACE and 5'-RACE reactions as described by the manufacturer (Life Technologies, Baltimore, MD). Introns of the echidna and platypus IGF2 (Fig. 1A) were amplified by long-template PCR (Roche Boehringer Mannheim, Indianapolis, IN) with the use of primers to putative flanking exons. Translational alignments of IGF2 domains A through D were performed with ClustalW followed by Boxshade-assisted shading (Biology Workbench, UCSD server). GenBank accession numbers for platypus and echidna IGF2 are AF339166 and AF339165, respectively.

**Determination of IGF2 imprinting in the platypus and echidna**

The imprint status of IGF2 in monotremes was determined using SNPs (single nucleotide polymorphisms). SNPs were screened for in monotreme IGF2 sequences by direct sequencing of the 3 exons and intron 2 (ABI 377 sequencer; PE Biosystems) (Fig. 1A). A single C→T transition polymorphism was detected in exon 3 of echidna IGF2 (nucleotide 548 of AF339165), whereas SNPs were not detected in 7 different platypuses. Echidnas that were polymorphic were selected for RT-PCR imprinting analysis (Kelsey and Reik, '98). Total RNA was extracted from tissues from the informative animals and reverse-transcribed as described above. PCR primers exon2F (5'-GGTGCCTTCAGCGAC) and exon3R (5'-CGGAGAGCGGGGTTGCCAC) designed to cross the spliced exon 2/exon 3 junction were used.

![Fig. 1. Monotreme IGF2. (A) Genomic structure of monotreme IGF2. PCR primers and the C/T SNP in exon 3 used to assess echidna IGF2 allelic expression are shown. (B) Species comparison of IGF2 domains A to D. Amino acid comparison demonstrates that IGF2 is highly conserved among chordates.](image-url)
to amplify the SNP from cDNA, while int2F2 (5'-CCCTCTCTCGCCCTGTCTAC) and exon3R were used with genomic DNA (Fig. 1A). PCR amplification was a single round of 30–35 cycles. The absence of contaminating genomic DNA during cDNA amplification was demonstrated by amplier size and by direct sequencing across the spliced exon–exon junction. Unbiased amplification of allelic variants was also demonstrated (data not shown). The imprint status of IGF2 was determined by comparing the genomic DNA and cDNA sequence at the polymorphic nucleotide position. All IGF2 allelic expression analyses were repeated 3 times, beginning with the cDNA synthesis step.

RESULTS AND DISCUSSION

Sequence and genomic structure of monotreme IGF2

IGF2 has been highly conserved in vertebrate evolution (Rotwein, '91). We initiated cloning of the echidna and platypus IGF2 by cross-species RT-PCR using four nondegenerate primers designed to conserved coding sequences of IGF2. These sequences were identified through visual inspection of an alignment of fish, avian, and mammalian IGF2 orthologues available in GenBank. Various combinations of the four CS-IGF2 primers yielded appropriately-sized PCR products from all mammals tested, including platypus and echidna. The complete cDNA sequence of platypus IGF2 was then obtained by 5'-RACE (i.e., rapid amplification of cDNA ends) and 3'-RACE.

IGF2 is a member of a family of homologous genes that include IGF1, insulin, and relaxin (Rotwein, '91). Our conclusion that the isolated platypus and echidna transcripts correspond specifically to IGF2 was based upon the following findings. Sequence alignment demonstrated that platypus IGF2 is highly homologous to orthologues from other vertebrate species (Fig. 1B). The mature platypus IGF2 peptide is 87% identical to artiodactyls and 85% identical to human and rodent IGF2. In contrast, the mature platypus IGF2 peptide is only 61%–64% identical to vertebrate IGF1 orthologues. The position of the stop codon in both the platypus and echidna IGF2 transcripts also corresponds exactly to that in other mammalian IGF2 orthologues.

The completed platypus IGF2 transcript revealed an uninterrupted open reading frame that contained a mature peptide consisting of domains A to D. It also contained a carboxyl terminal E domain that is not present in the mature IGF2 peptide. Consistent with the known large divergence of the IGF2 E domain across vertebrates (Rotwein, '91), the E domain encoded by the monotreme transcripts is only 42%, 37%, and 36% identical to those in therian mammals, birds, and fish, respectively. Moreover, there is no homology between the monotreme IGF2 E domain and the carboxyl termini of other members of the IGF family. Thus, the presence of a moderately conserved IGF2 E domain in monotremes is diagnostic for IGF2.

Characterization of the genomic structure of platypus and echidna IGF2 uncovered exon–intron junctions analogous in position and intron phase to other IGF2 orthologues (Fig. 1A) (Rotwein, '91). Specifically, there are three monotreme IGF2 coding exons, interrupted by a proximal intron of 5 kbp and a distal intron of 2 kbp. The first intron disrupts B-domain glycine 25 after the first nucleotide of the codon (intron phase 1); the second intron interrupts the E domain coding sequence before D-Y-Q ... (intron phase 0). The position and phase of the monotreme IGF2 introns are therefore identical to those present in IGF2 of other vertebrates. Thus, the recovered platypus and echidna transcripts can be unambiguously classified as IGF2.

Imprint status of monotreme IGF2

Platypuses and echidnas can only be bred in captivity with great difficulty, and such animals are not available for scientific studies. IGF2 imprinting studies in these species therefore require the availability of wild animals that are polymorphic at this locus. The use of wild animals carries the benefit of not introducing unknown experimental influences on imprinting, which occurs with the generation of interspecific hybrids and transgene manipulation (O’Neill et al., ’98; Vrana et al., ’98). A C→T IGF2 transition polymorphism was identified in the 3’-UTR of the echidnas while polymorphisms were not found in the screened platypus samples.

Therefore, to determine the imprint status of IGF2 in monotremes, RNA was prepared from intestine, liver, kidney, and spleen of two informative echidnas. cDNA was synthesized by reverse transcription of DNase-treated RNA, and cDNA-PCR was primed with oligonucleotides that hybridize in exons separated by a 2 kbp intron (Fig. 1A). The amplification of a spliced IGF2 transcript was confirmed by amplimer size and sequencing across the spliced exon–exon junctions. Sequenc-
ing the echidna IGF2 transcript amplimers demonstrated biallelic IGF2 expression in all tissues examined (Fig. 2). Our detection of biallelic IGF2 expression in multiple echidna tissues, coupled with the absence of evidence for polymorphic IGF2 imprinting in any previously investigated mammalian species (O’Neill et al., 2000), demonstrates that IGF2 is not imprinted in monotremes.

Fig. 2. Allelic expression of IGF2 in the echidna. DNA of echidna A and B contain a C/T transition polymorphism (arrow) in IGF2 exon 3. RT-PCR analysis of spliced IGF2 mRNA transcripts in the spleen of echidna A and the intestine, kidney, liver, and spleen of echidna B demonstrate equal biallelic expression of parental alleles.
Phylogenetic distribution of genomic imprinting

Biallelic expression of IGF2 in monotreme mammals parallels our earlier finding that M6P/IGF2R is not imprinted in monotremes. Thus, the imprinting of two genes critically involved in embryonic growth—IGF2 and M6P/IGF2R—is present in marsupial and eutherian species but is absent in monotremes, birds, and presumably all other chordates (Fig. 3). Because M6P/IGF2R is divergent among mammals in both its function and imprint status (Killian et al., 2000), it is not the best gene for determining the phylogenetic origin of genomic imprinting. In contrast, IGF2 is imprinted in all marsupials and eutherian mammals investigated. The combined imprint profile of M6P/IGF2R, an evolving growth factor receptor (Jirtle, '99; Killian et al., 2000), and IGF2, a highly conserved growth factor (Fig. 1A) (Rotwein, '91), indicates that the imprinting of genes principally involved in regulating gestational growth originated in Jurassic/Cretaceous therian mammalian ancestors (Fig. 3).

Monotreme and marsupial reproduction are in many ways more akin than those of marsupials and eutherians (Table 1) (Griffiths, '78, '99; Tyndale-Biscoe and Renfree, '87; Hughes, '93; Hughes and Hall, '98). This raises the question of what reproductive distinction in therian mammals drove the evolution of genomic imprinting. The most remarkable qualitative difference between monotreme and marsupial gestation is the loss of the egg shell 2/3 of the way through gestation in marsupials. A vascularized yolk-sac placenta then establishes an intimate relationship with the uterine epithelium in the case of the opossum (Krause and Cutts, '85). Among mammals, the unshelled apposition of embryo and uterine wall is unique to therians and might therefore escalate interparental conflicts to the point where imprinting is selected.

Although the quantity of monotreme and marsupial maternal investments appear equally contentious from the mother’s and father’s perspectives, evolutionary change in response to selective pressures is neither progressive nor predictable. Rather, evolution occurs by way of contingent and fortuitous events (Gould, '94). The substrates for natural selection are random genetic and epigenetic events coupled with an appropriate genetic background and selective pressures. IGF2 and M6P/IGF2R imprinting in therian mammals may therefore have evolved in the context of unique but still undiscovered epigenetic, genetic, and environmental convergences unknown to prototherian mammals. Thus, despite the ostensible presence of adequate interparental conflict, the imprinting of select loci may have been histori-
cally impossible in monotremes. This postulate is supported by our recent finding that $M_6P/IGF2R$ imprinting only evolved subsequent to the receptor acquiring the ability to bind IGF2 (Killian et al., 2000).

Among Amniotes, gestation of developing offspring is not limited to mammals: studies of squamate reptiles reveal that viviparity has evolved convergently upwards of 100 times (Blackburn, 2000). The genetic and physiological adaptations to viviparity are diverse, and universal mechanisms and common pathways are unlikely. Fortuitous genetic and epigenetic events in therian mammalian ancestors may have permitted the escalation of an interparental arms race through the imprinting of gestation-regulatory genes in the IGF-pathway. While IGF-pathway imprinting appears limited to therian mammals, entirely unknown interparental genetic conflicts may yet be identified in reptiles and monotremes.

Beyond reproductive physiology, the imprint status of $IGF2$ in the three living monophyletic divisions of mammals also helps resolve their sister relationships. The Theria hypothesis argues for the early divergence of monotremes from a separate taxon comprised of marsupial and eutherian mammals (Marshall, ’79), whereas the heterodox Marsupionta hypothesis proposes a close kinship between monotremes and marsupials exclusive of eutherians (Gregory, ’47). Imprinting of both $IGF2$ and $M_6P/IGF2R$ in therian but not prototherian mammals provides further support of the Theria hypothesis of mammalian taxonomy (Fig. 3) (Killian et al., 2000, 2001; Luo et al., 2001).

CONCLUSIONS

We have identified the ancestral mammalian origin of parental imprinting of growth regulatory genes in the IGF-pathway, and demonstrated that both $IGF2$ and $M_6P/IGF2R$ are imprinted in therian but not in prototherian mammals. It is still unknown, however, if all mammalian imprinted genes evolved in a common ancestor to therian mammals or if some genes became imprinted after marsupials diverged from eutherians. Thus it is still essential to establish the phylogenetic distribution of imprinting for other genes, most notably those that regulate behavior such as $PEG1/MEST$ and $PEG3$ (Lefebvre et al., ’98; Li et al., ’99).

ACKNOWLEDGMENTS

The authors thank M. Renfree for helpful discussions on mammalian reproduction and the Duke University DNA analysis facility for DNA sequencing. For additional information on genomic imprinting, visit http://www.geneimprint.com.

LITERATURE CITED


